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Comparison of alternative extraction methods for secretome profiling in human hepatocellular carcinoma cells

CAO Jing[1], SHEN ChengPin[1], ZHANG Jun[1], YAO Jun[1], SHEN HuaLi[1], LIU YinKun[1,2], LU HaoJie[1] & YANG PengYuan[1*]

[1]*Institutes of Biomedical Sciences and Department of Chemistry, Fudan University, Shanghai 200032, China;*
[2]*Liver Cancer Institute Zhongshan Hospital, Fudan University, Shanghai 200032, China*

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Secreted proteins are important sources for early detection and diagnosis of disease, and as such have received considerable attention. The extraction of low concentration proteins from large volumes of culture media, which are rich in salts and other compounds that interfere with most proteomics techniques, presents a problem for secretome studies. Ultrafiltration, precipitation, and dialysis are three major extraction methods that can be used to overcome this problem. The present study for the first time, compared the merits and shortcomings of these three methods, without bias. Centrifugal ultrafiltration provided the best extraction efficiency, and precipitation provided the highest number of identifiable proteins. The three methods yielded closely related, but different, information on the secretome; thus, they should be considered complementary or, at least, supplementary methods. Three hundred and sixty unique proteins were identified, including 211 potential secreted proteins. Compared with previous studies, this study also identified 42 new secreted proteins. The present study not only offers a reference for the selection of secretome extraction methods, but also expands the secretome database for the investigation of hepatocellular carcinoma.

hepatocellular carcinoma, secretome, protein extraction, ultrafiltration, precipitation, dialysis, LC-MS/MS

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The secretome has a pivotal role in many biological processes, including signal transduction, immune defense, cell-cell interaction, cancer cell invasion, and metastasis. Many proteins overexpressed in tumors are secreted proteins [1–3]. For example, osteopontin, a secreted phosphoprotein whose overexpression correlated with the metastatic potential of primary hepatocellular carcinoma (HCC) and with invasiveness of liver tumor-derived cell lines *in vitro* [4]. Metastasis remains the cause of 90% of deaths from solid tumors [5]. HCC is no exception, and is one of the most common and aggressive human malignancies [6]; its high mortality rate is mainly the result of intra-hepatic metastases.

Thus, the analysis of a cell's secretome could be valuable in diagnosis and prognosis. However, proteins are usually secreted at low concentrations into the culture media, which makes their recovery difficult. Currently, there are three major methods for the purification and concentration of secretory proteins: ultrafiltration [7–9] with molecular-weight cut-off membranes, precipitation [10–12] with organic solvents, e.g., trichloroacetic acid (TCA), and dialysis [13–15] with cut-off membranes followed by drying using vacuum centrifugation. Each of these methods has its own disadvantages, and no single one can capture all proteins secreted into the culture media. Accordingly, we used all three methods to investigate the secretome of the human

*Corresponding author (email: pyyang@fudan.edu.cn)

HCC cell line, HCCLM3. Accompanied by nano-LC-ESI-MS/MS analysis, the three methods were compared in terms of their merits and shortcomings.

1 Materials and methods

1.1 Cell culture

The HCCLM3 cell line had been established previously [16]. Cells were grown in DMEM culture medium containing 10% fetal bovine serum until 60%–70% confluence was achieved [17]. The cells were stringently and gently washed, twice with Dulbecco's phosphate buffered saline with calcium and magnesium (DPBS), and once with serum- and phenol red-free DMEM (conditioned medium, CM). The cells were then incubated in CM at 37°C. After 24 h, the CM containing secreted proteins was collected and centrifuged at 1000×*g* for 5 min (4°C), and then filtered using a 0.45 μm filter (Millipore, Bedford, MA, USA) to pellet detached cells and large debris. The supernatant was collected and then centrifuged for 1 h at 100000×*g* (4°C) to pellet smaller debris and vesicles. TFA (0.1%) was immediately added to the final supernatant, which was stored at −80°C. The addition of TFA lowered the pH (<4) of the culture supernatants, thus reducing the activity of many proteases.

1.2 Preparation of secretory proteins

The proteins present in the culture supernatants were extracted by various procedures. Twenty milliliters of culture supernatants was used for each method. All the treatments were done at 4°C. The concentrations of the extracted proteins were measured by the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA).

1.2.1 Centrifugal ultrafiltration

The culture supernatants were concentrated using centriprep centrifugal filter devices with Ultracel YM 3000 Daltons MWCO membrane (Millipore, Bedford, MA, USA) at 3000×*g* for 6 h. The resulting concentrate was collected.

1.2.2 Precipitation

The culture supernatants were precipitated using a previously described method [18], with minor modifications. Sodium lauroyl sarcosinate (NLS) at 0.5% final concentration was introduced into the CM solution. After mixing, TCA was added to a final concentration of 12%, and the solution was precipitated on ice for 2 h. The mixed protein-detergent precipitate was collected by centrifugation (10000×*g*, 10 min, 4°C). The supernatant was carefully removed, 2 mL of tetrahydrofuran (THF) (pre-cooled in ice) was added to the pellet and vortexed until the pellet dislodged from the bottom of the tube and dissolved almost completely. Centrifugation was carried out as described above. The supernatant was removed, and the nearly invisible pellet was washed again with 2 mL of THF. Finally, the pellet was redissolved in 0.4 mL extraction solution with the help of a sonicator bath (30 min extraction).

1.2.3 Dialysis

The culture supernatants were dialyzed against water (molecular mass cut-off 3500 Da; Spectrum, CA, USA) for 48 h and concentrated using a SpeedVac (CHRIST, Germany).

1.3 Western blot analysis

Proteins (30 μg) of the culture supernatants and cell extracts were analyzed by Western blotting, as previously described [19]. Proteins were separated on SDS-PAGE, transferred onto PVDF membranes, and probed with antibodies against β-tubulin, followed by secondary antibody conjugated with horseradish peroxidase. The blot was developed using the Western lightning chemiluminescence reagent (GE Healthcare, USA).

1.4 In-solution digestion

The extracted proteins were dissolved and heated at 100°C for 10 min. After allowing the sample to cool to room temperature, dithiothreitol (DTT) was introduced into the solution at a final concentration of 10 mmol L^{-1}, and the sample was incubated at 57°C for 30 min. To prevent disulfide bond formation, cysteine residues were modified by alkylation with iodoacetamide (20 mmol L^{-1} final concentration) for 30 min in the dark, at room temperature. The reaction was quenched by the addition of DTT at half of the molar concentration of the iodoacetamide for 10 min. After iodoacetamide deactivation, the sample solution was diluted 10 folds with 50 mmol L^{-1} NH_4HCO_3 buffer. Trypsin was added to the sample (1∶50) to digest the proteins overnight (at 37°C). All digested peptide mixtures were dried using vacuum centrifugation and stored at −20°C until analysis by mass spectrometry.

1.5 LC-MS/MS analysis

All digested peptide mixtures were resuspended with a solution containing 5% acetonitrile and 0.1% formic acid, separated by on-line nanoLC and analyzed by electrospray tandem mass spectrometry. The experiments were performed on an LC-20AD system (Shimadzu, Tokyo, Japan) connected to an LTQ Orbitrap mass spectrometer (Thermo Electron, Bremen, Germany) equipped with a nanoelectrospray ion source (Michrom Bioresources, Auburn, USA). The separation of the peptides took place in a 10-cm column (75-μm inner diameter; New Objective, Woburn, USA) packed with 5 μm Biobasic™ C18.

The peptide mixtures were injected onto the trap-column

with a flow of 60 μL min^{-1} and subsequently eluted with a gradient of 5%–45% solvent B (95% acetonitrile in 0.1% formic acid) over 90 min. The peptides were then injected into the mass-spectrometer at a constant column-tip flow rate of ~300 nL min^{-1}. Eluted peptides were analyzed by MS and data-dependent MS/MS acquisition, selecting the eight most abundant precursor ions for MS/MS with a dynamic exclusion duration of 1 min. Two biological replicates were performed for each concentration method, and each biological replicate was analyzed four times.

1.6 Database searching

The mass spectra were searched against the human International Protein Index (IPI) database (IPI human v3.35 fasta with 62322 entries) using the Bioworks software (Version 3.3.1; Thermo Electron Corp.), based on the Sequest algorithm. The search parameters included (i) precursor ion mass tolerance less than 0.005%; (ii) fragment ion mass tolerance less than 1 Da; (iii) up to three missed tryptic cleavages allowed; and (iv) amino acid modifications cysteine carboxyamidomethylation (plus 57.05 Da), and methionine oxidation (plus 15.99 Da). The corresponding reversed sequence database was used to generate score criteria that yielded an estimated FP (False Positive) rate of 5% (precision of 0.95). To minimize false positives, all output results were combined together using in-house software to generate score criteria: The cross-correlation scores (Xcorr) of matches were greater than 2.81 and 3.39 for charged state 2 and 3 peptide ions, respectively. To obtain reliable protein identification, only peptides with a ΔCn score above 0.1, and whose ranks of the primary scores (Rsp) were less than 4, were used [20,21]. In addition, proteins were identified from two or more distinct peptides, which could be considered as a high-confidence identification.

1.7 Bioinformatics analysis

The theoretical isoelectric point (pI), molecular weight (MW) and GRAVY (grand average of hydropathy) values were calculated using software developed in-house. The secretion pathways of the identified proteins were predicted by SecretomeP [22] (free online at http://www.cbs.dtu.dk/services/SecretomeP). The mapping of putative transmembrane domains in identified proteins was carried out using the transmembrane hidden Markov model (TMHMM) algorithm, available at http://www.cbs.dtu.dk/services/TMHMM [23].

2 Results and discussion

2.1 Verification of secreted proteins by Western blotting

To reduce contaminants from cytoplasmic proteins and harvest pure secreted proteins, all the procedures descried in Cell culture were carried out stringently. In addition, we examined the distribution of β-tubulin, an abundant cytoskeletal protein in the two fractions. As shown in Figure 1, β-tubulin was clearly detected in the total cell extracts, but not in the CM. These observations imply that proteins recovered from the culture supernatants were not the result of cell death.

2.2 Comparison of extraction methods

In terms of handling, dialysis was the simplest, but the most time-consuming (2 d). Ultrafiltration was the second most time-consuming method: It suffered from blockage of the column during centrifugation and the loss of low molecular weight protein. However, using precipitation concentration, the resulting precipitates were difficult to dissolve for further analysis, even if a high concentration of the urea solution was used. In terms of efficiency of extraction, the protein yields of these three methods were different. From 20 mL of culture supernatants, 70, 92.5, and 32.5 μg proteins were extracted by precipitation, centrifugal ultrafiltration, and dialysis, respectively. Thus, centrifugal ultrafiltration appeared to be the best in terms of extraction efficiency.

As shown in Figure 2, 360 unique proteins were identified in total. 206, 290, and 157 proteins were yielded by ultrafiltration, precipitation, and dialysis, respectively. Among them, 106 proteins were identified by all three

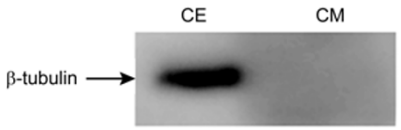

Figure 1 β-tubulin identification by Western blotting with the anti-β-tubulin antibody. Proteins (30 μg) from cell extracts (CE) and conditioned medium (CM) were analyzed.

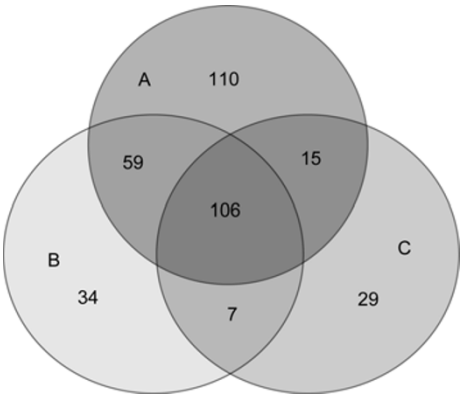

Figure 2 A total of 360 unique proteins were identified by different concentration methods. Ultrafiltration, precipitation, and dialysis yielded 206, 290, and 157 proteins, respectively, with 106 proteins being identified by all three methods. 34, 110, and 29 proteins were uniquely identified in extracts prepared by ultrafiltration, precipitation, and dialysis, respectively.

methods. Ultrafiltration, precipitation, and dialysis yielded 110, 29, and 34 proteins, respectively, which were unique to that method. Thus, based on the number of proteins identified, precipitation had an advantage and these three concentration methods could supplement each other. The concentration method that produced each identified protein is indicated in Appendix Table S1 in the electronic version.

To assess possible analytical bias of each method, we calculated the MW, pI, and GRAVY values of each protein, based on its primary amino acid sequence. The 290 proteins harvested by precipitation showed a typical molecular weight distribution (8–630 kD), with a maximum of 28% below 30 kD (Figure 3A); the smallest MW was 8 kD. By contrast, 19% of proteins from the dialysis method were below 30 kD. Thus, for the enrichment of low molecular weight proteins, the precipitation method is superior to the other two methods. Figure 3B shows the distribution of the pI values for the three methods: The proteins cover a wide pI range and even very basic proteins (up to a pI of 11.86) can be identified. The distribution of the pI values for the 360 identified proteins is consistent with previous descriptions of the Golgi compartment [24]. Figure 3C shows that the secretome from precipitation presents the most extensive distribution of GRAVY values and includes more hydrophilic proteins. The overall mean of the GRAVY values was –0.386 between the cytoplasm and endoplasmic reticulum, the values of which were –0.50 and –0.075, respectively, in a previous study. Of all proteins studied, 5% had positive GRAVY values indicating a hydrophobic nature.

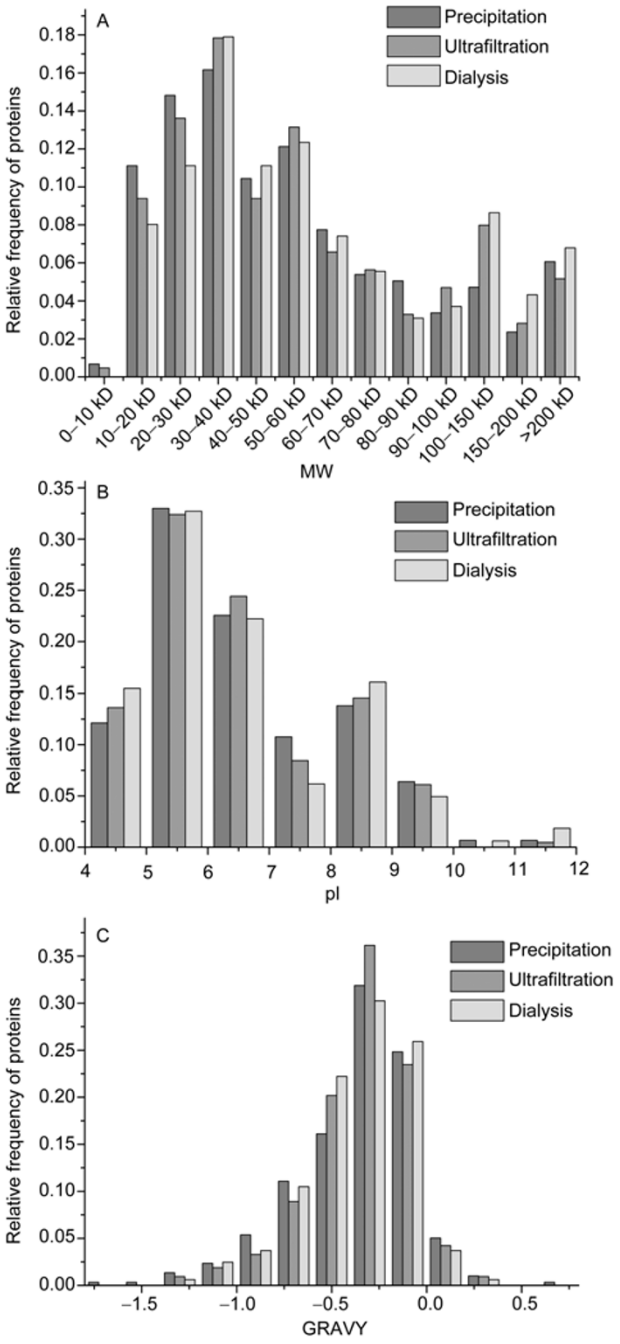

Figure 3 Comparisons of protein distributions based on their physiochemical characteristics. A, MW; B, pI; C, GRAVY among the proteins identified from the three methods.

2.3 Data analysis

2.3.1 Secretory pathway categories of identified proteins

The identified proteins were further analyzed using bioinformatics software to predict protein secretion pathways. Seventy-eight proteins were predicted by SecretomeP to be released through the nonclassical secretory pathway. Ninety-seven proteins were predicted to be secreted by the classical secretory pathway, which is characterized by the presence of a signal peptide and absence of transmembrane domains [25,26]. In addition, 36 integral membrane proteins were predicted by TMHMM that could not be categorized in the classical or nonclassical secretion pathways. Collectively, these analyses predicted that at least 58.6 % (211/360) of the identified proteins could be released into the CM of cultured cancer cells via different mechanisms. These 360 classified proteins are all displayed in Appendix Table S1 in the electronic version.

2.3.2 Identification of known or novel secretory proteins

Altogether, from the three methods, 360 proteins were confidently identified. Although 360 is not a large number, the efficiency of identification in our study was better than that in a previous study [9], which analyzed a human hepatoma cell, HepG2, using two-dimensional liquid chromatography coupled with tandem mass spectrometry. In that study, only 86 proteins were identified. In addition, in our previous work [27,28], 187 secretory proteins were identified using a glycoproteomics strategy, and 872 secretory proteins were identified using a novel nanozeolite-driven enrichment approach, followed by SDS-PAGE and LC-MS/MS. In the present work, 211 secretory proteins were identified, of which 42 were newly identified, compared with our two previous studies. Furthermore, compared with the dataset

Rank0 of SPD, a web-based secreted protein database [29], this work led to identification of some known, as well as novel, secreted proteins. Among the 42 newly identified secretory proteins, 11 proteins were known secreted proteins in UniProtKB/Swiss-Prot; however, the other 31 proteins were not annotated as secreted proteins in that database. Thus, the 31 secretory proteins were newly identified in this study, and are all marked with symbols in Appendix Table S1 in the electronic version.

3 Conclusion

Comprehensive recovery of secreted proteins is a very challenging task using available extraction strategies. All three strategies applied in this study are widely used. No previous study has compared all three methods to determine which is the best. There was one previous study [18] by Chevallet et al., which compared ultrafiltration and precipitation, but not the dialysis method. They concluded that the lauroyl sarcosinate-TCA precipitation method was better, and did not mention any advantages of ultrafiltration. Our study is the first to compare all three extraction methods, without bias. We found that (i) ultrafiltration generated a higher protein yield than precipitation; (ii) for the enrichment of low molecular weight proteins and quite hydrophilic proteins, precipitation is superior to the other two methods; and (iii) using the number of proteins identified, we concluded that precipitation has the advantage, but these three concentration methods could complement each other.

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